J. clin. Path., 1975, **28**, 722-727

Effect of human lysozyme (muramidase) on potassium handling by the perfused rat kidney

A mechanism for renal damage in human monocytic leukaemia

D. Y. MASON, D. T. HOWES, C. R. TAYLOR, AND B. D. ROSS

From the Gibson Laboratories, Radcliffe Infirmary, Oxford

SYNOPSIS Purified human lysozyme (muramidase) stimulated potassium excretion by the isolated perfused rat kidney. Lysozyme is filtered and reabsorbed, without a tubular maximum. Over 90% of lysozyme filtered is retained within the kidney; 50% was recovered by enzymic assay and histologically localized to the proximal tubular cells. Hypokalaemia seen in some patients with myelomonocytic leukaemia may be directly attributed to an elevated circulating lysozyme level.

Osserman and Lawlor (1966) reported 10 consecutive cases of monocytic and myelomonocytic leukaemia in all of whom large quantities of urinary lysozyme (muramidase)[1] were demonstrable. Low serum potassium values, together with increased levels of urinary potassium, were noted in several of these patients, and a possible causal relationship between muramidasuria and renal potassium loss was proposed.

Subsequent work has strengthened the concept that muramidase induces the kidney to excrete increased amounts of potassium. However, the mechanism of this phenomenon is not clear. Pickering and Catovsky (1973) found hypokalaemia in almost half of the 25 patients with myelomonocytic and monocytic leukaemia they studied. One patient, investigated in more detail, had renal potassium wasting but no other evidence of tubular dysfunction. Muggia, Heinemann, Farhangi, and Osserman (1969) suggested that, apart from renal potassium loss, there were other proximal tubular defects in two leukaemic patients out of the three investigated. There is some doubt, therefore, whether the tubular derangement in monocytic leukaemia is selective for potassium handling or is more generalized. Wiernik and Serpick (1969) point out that the tubular dysfunction may itself be responsible for the increased urinary excretion of muramidase.

The association of muramidase and renal potassium loss may be direct or indirect; assays of serum cortisol before and after ACTH stimulation, of serum renin, and of urinary aldosterone were normal (Pickering and Catovsky, 1973; Muggia *et al*, 1969). This strengthens the view that lysozyme may have a direct effect upon the kidney.

In order to examine the effect of purified human muramidase on renal potassium excretion an improved method of isolated rat kidney perfusion has been used. Inulin clearance (GFR), potassium excretion, and muramidase handling were studied in the presence and absence of added muramidase.

At concentrations of muramidase which are found in human myelomonocytic leukaemia, there was a significant increase in urinary potassium excretion.

[1]In 1964 the Commission on Enzyme Nomenclature recommended: E.C. 3.2.1.17. Systematic name: mucopeptide N-acetylmuramylhydrolase; recommended trivial name: mucopeptide glucohydrolase, lysozyme; other name *not* recommended: muramidase

Received for publication 22 February 1975.

Methods

MURAMIDASE

Human muramidase (MW 17 000) was isolated from the urine of two monocytic leukaemic patients according to the method of Johansson and Malmquist (1971). After absorption of muramidase from the pooled urine onto CM-50 Sephadex, the enzyme was eluted with 2 M tris buffer, dialysed against distilled water, and then freeze dried. On acrylamide gel electrophoresis the preparation was homogeneous. However, by immunodiffusion in agar against anti-IgG and anti-light chain sera, traces of immunoglobulin could be detected. Consequently batches of the freeze-dried preparation were further purified by passage through Sephadex G-75.

Perfusion experiments were performed with both the initial preparation and also with the G-75 purified material. No difference in the effects of the

two preparations on kidney function could be detected, and the results obtained with both materials have been considered together.

IMMUNOHISTOLOGICAL DEMONSTRATION OF HUMAN MURAMIDASE

This technique, which involves a 'sandwich' of anti-muramidase, swine anti-rabbit serum protein, and immune complexes of peroxidase/rabbit anti-peroxidase, has been described and shown to be specific for muramidase (Mason and Taylor, 1975).

ASSAY OF MURAMIDASE IN PERFUSING MEDIUM AND URINE

Two assay techniques were used: the lyso-plate method of Osserman and Lawlor (1966); and a turbidometric technique in which the increase in transmission at 436 nm of a suspension of *Micrococcus lysodeikticus* (0·30 mg/ml in pH 7·0, 0·06M phosphate buffer), after the addition of muramidase, is measured in an SP8000 recording spectrophotometer. The micrococcus for both assays was obtained from Sigma Biochemicals. The techniques have been shown to give comparable results, and this was confirmed in the course of these experiments (Zucker *et al*, 1970).

KIDNEY PERFUSION

An isolated perfusion technique for rat kidney was used in the present experiments (Ross *et al*, 1973). The haemoglobulin-free perfusion medium consists of 6·7g% dialysed bovine serum albumin, fraction V, in Krebs-Henseleit saline, gassed with 5% CO_2:95% O_2. Glucose (5 mM) was the only added substrate. In individual experiments, muramidase was added after a control period of 40 minutes' perfusion. ^{14}C inulin clearance (GFR), urinary Na^+ and K^+ were determined in successive 10-minute periods. Samples for the analysis of muramidase content in urine and perfusion medium were taken at 10-minute intervals after addition of muramidase. Each kidney thus served as a control for the determination of sodium reabsorption and potassium excretion in the presence and absence of muramidase. Finally, the kidney was divided into samples for the determination of wet weight/dry weight ratio and for the chemical determination of muramidase content, and a portion was fixed in 10% formalin for histological examination and detection of muramidase by the histochemical method.

Results

MURAMIDASE HANDLING BY THE PERFUSED KIDNEY

Normal human plasma muramidase levels are less than 10 μg/ml, while levels of 30 to 100 μg/ml or more are found in leukaemic patients (Osserman and Lawlor, 1966; Pickering and Catovsky, 1973; Muggia *et al*, 1969). The effect of concentrations between 1 μg/ml and 600 μg/ml were studied in the present experiments. Addition of muramidase to the perfusion medium resulted in the recovery of muramidase at increased concentration in the urine, and the disappearance of muramidase from the medium at a linear rate. Figure 1 represents an experiment in which 75 μg/ml muramidase were added after 45 minutes of perfusion. The rate of disappearance of muramidase from the medium greatly exceeded the rate of excretion into the urine, indicating that muramidase was either metabolized by, or concentrated within the kidney.

At increasing concentrations of muramidase, tested in individual kidneys, muramidase excretion increased linearly as the filtered load (GFR × muramidase concentration in medium) increased. Reabsorption of muramidase, which was 96·5% at a medium content of 1 μg/ml muramidase, fell to 62·8% when the filtered load was 94 μg/minute (medium muramidase concentration 600 μg/ml) (fig 2). However, no tubular maximum for muramidase reabsorption was observed even at these very high medium concentrations.

The rate at which muramidase was removed from the medium, uncorrected for the urinary excretion,

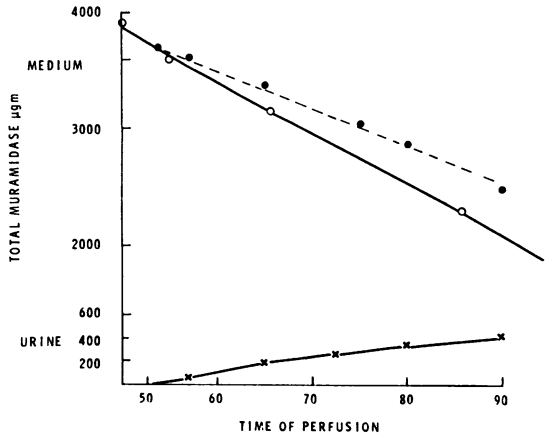
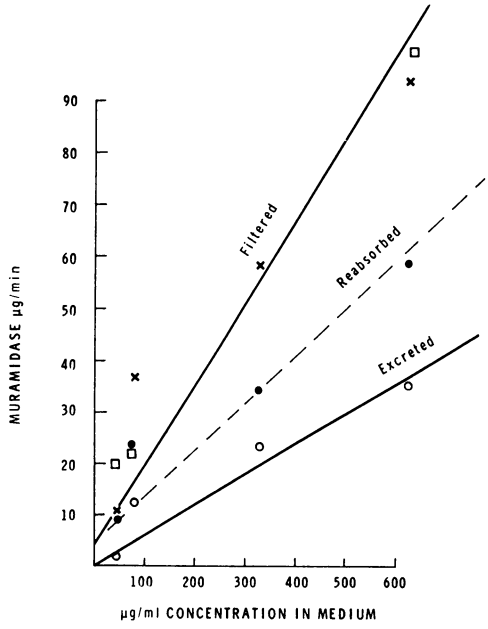


Fig 1 *Removal of added muramidase from the medium by perfused rat kidney. Muramidase was added to the medium at a concentration of 75 μg/ml after 50 minutes of perfusion. Total muramidase was calculated from the final volume of perfusion medium.* ×———× *muramidase recovered in urine (total);* ○———○ *muramidase removed from medium (total);* ●- - -● *muramidase removed but not recovered (total).*



was also dependent upon the medium concentration, and at concentrations between 10 and 600 µg/ml of medium was close to the calculated filtration rate for muramidase in these experiments. This may be taken as evidence that muramidase is filtered and largely reabsorbed, and that active secretion of muramidase does not occur.

RECOVERY AND LOCALIZATION OF MURAMIDASE WITHIN THE KIDNEY

Two kidneys were assayed for muramidase content at the end of perfusions with 75 µg/ml muramidase. A crude homogenate of kidney in phosphate buffer (1:4) was sonicated 3 × 10 sec, and muramidase

Fig 2 *Effect of increasing concentration on renal handling of muramidase. Each point represents a single kidney perfusion (mean of 4 or 5 clearance periods) conducted at a different concentration of muramidase in the medium. Filtered load is calculated from clearance of ^{14}C inulin. □———□ rate of muramidase removal from medium (cf fig 1).*

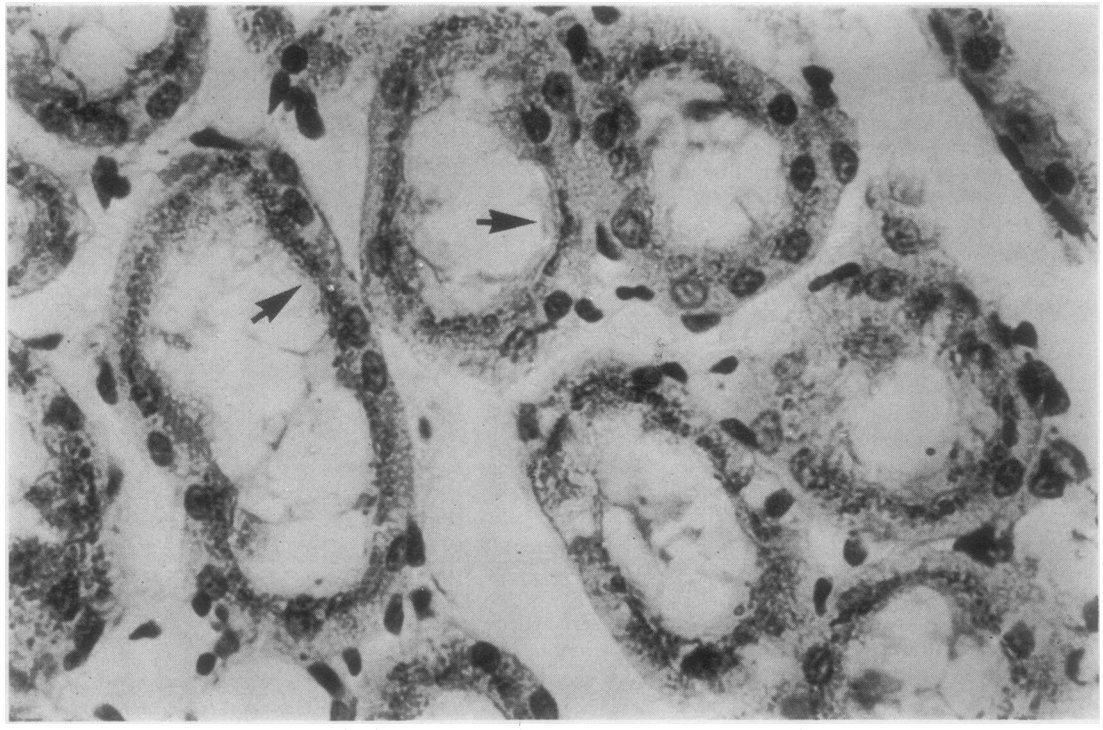


Fig 3 *Perfused rat kidney, stained for human muramidase. Perfusion was continued for 40 minutes after the addition of human muramidase (30 µg/ml) to the medium. Material staining as human muramidase is seen beneath the luminal pole of the majority of proximal tubular cells. Arrows point to regions where this reaction is particularly clearly seen (as a dark granular deposit). Haematoxylin counterstain × 400.*

was determined in the whole homogenate. The concentration of muramidase in each kidney was four to five times the final concentration in the perfusion medium, when the content of endogenous (rat) muramidase was subtracted from the total kidney content. Of somewhat over 1000 μg muramidase removed from the medium and not recovered in the urine, 570 μg muramidase were recovered in the kidney substance.

Histological localization of human muramidase within the kidney was achieved by the specific antibody technique (methods) and showed intense staining due to muramidase beneath the luminal pole of the cells lining the proximal tubule (fig 3). Little or no material was seen in the glomeruli or the distal tubular cells, but some positive staining material remained in the lumen of the collecting tubules and ducts. Rat muramidase did not stain with this technique, and a control kidney showed no reaction when perfused with muramidase-free medium and subjected to the same histological procedures.

EFFECT OF MURAMIDASE ON RENAL ELECTROLYTE HANDLING

In control perfusions, without muramidase, GFR was approximately 0·6 ml/min and fell slightly during 60 minutes of perfusion. Sodium reabsorption was 93-95% of the filtered load. Potassium handling by this preparation has been characterized (Silva *et al*, in press) and in the control perfusion, with glucose as substrate, potassium is excreted without evidence of net secretion; U/P potassium/U/P inulin is normally 0·3-0·6 at medium potassium concentrations between 4·0 and 6·0 mEq/l.

When muramidase was added to the perfusion medium there was an immediate increase in the excretion of potassium in the urine, an effect which was seen in the first 10 minutes collection period and persisted for the remaining 30-60 minutes of perfusion. $U/P_K/U/P_{In}$ increased from 0·55 ± 0·10 (6 experiments) in the control period to 0·72 ± 0·01 (6) in the collection periods after addition of muramidase. In some experiments, U_KV increased too, but often this was obscured by the gradual decline in GFR. Figure 4 shows the effect of adding 30 μg/ml muramidase to the perfusion medium: the $U/P_K/U/P_{In}$ ratio increased and excretion of potassium, corrected for 1 ml of GFR at each collection, increased accordingly.

The stimulation of potassium excretion was observed at all concentrations of muramidase between 1 μg/ml and 600 μg/ml to a similar extent (fig 5), but even the highest doses of muramidase

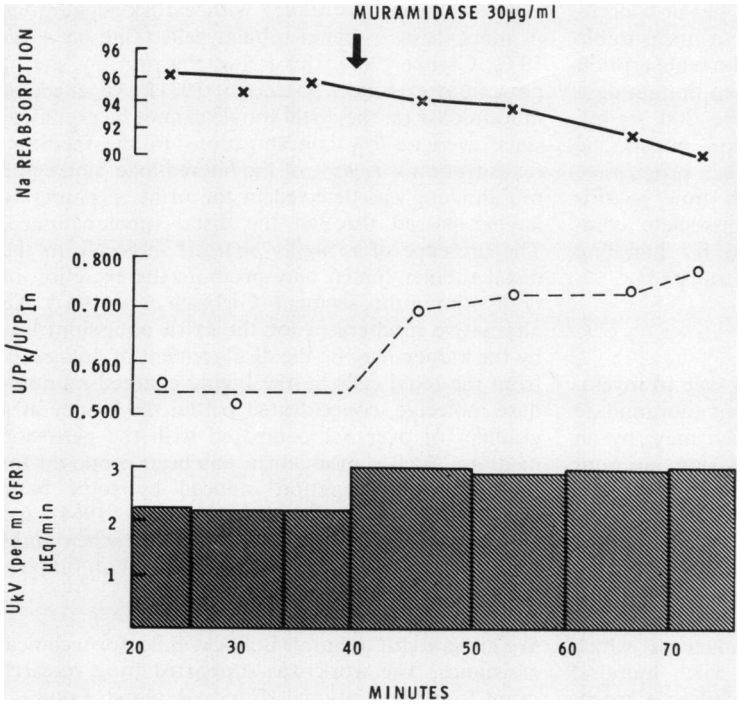


Fig 4 *Effect of human muramidase on renal handling of potassium. Muramidase (30 μg/ml) was added after 40 minutes of perfusion, during which potassium excretion was constant. The rate of decline in % Na reabsorption with time was not different from the control without muramidase.*

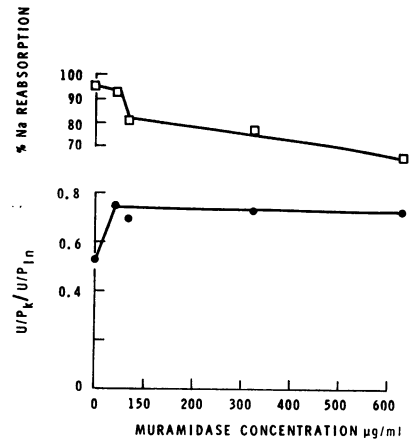


Fig 5 *Effect of increasing concentration of muramidase on potassium excretion and % Na reabsorption. Each point is the mean of four or five clearance periods in one or two perfused kidneys after addition of muramidase at the concentrations indicated in each kidney.*

did not produce net secretion of potassium, that is, $U/P_K/U/P_{In}$ did not exceed 1.

At the lowest concentrations of muramidase, sodium reabsorption was unaffected, but at concentrations over 75 µg/ml there was a measurable inhibition of Na^+ reabsorption. Sodium reabsorption was only 70% of the filtered load when muramidase was present at concentrations above 300 µg/ml, suggesting that at this concentration nonspecific toxic effects appear. However, the progressive decrease in Na^+ reabsorption with time in this preparation makes it difficult to dissociate completely the effect of muramidase on K^+ handling from an inhibitory effect on sodium transport.

Discussion

The basis of the hypokalaemia often seen in myelomonocytic leukaemia is obscure, but muramidase has been proposed as an agent which may, by an effect on the tubular handling of potassium, account for some or all of the extra potassium excretion seen in these patients. The present experiments show that muramidase from the urine of leukaemic patients does have the effect of increasing potassium excretion by the isolated rat kidney. This model permits the study of renal potassium handling in the absence of the many humoral influences which normally control tubular function. Such humoral mediation may, therefore, be excluded as a mechanism of hypokalaemia in myelomonocytic leukaemia (Pickering and Catovsky, 1973; Muggia *et al*, 1969).

Human muramidase, a protein of molecular weight 15-17 000, was filtered as expected (Hansen *et al*, 1972). The use of 'foreign' muramidase enables the concentration of muramidase within the proximal tubular cell of the rat kidney to be seen as the reabsorption of filtered protein, rather than renal synthesis of muramidase as has been suggested (Balazs and Roepke, 1966). In a recent study using perfused rat kidney, Maack and Sigulem (1974) have demonstrated that rat muramidase is treated as a filtrable molecule, with characteristics of renal handling very close to those found with human muramidase in the present study. Only when the muramidase load exceeded 1000 µg/min, over 10 times the level of human muramidase tested in the present study, was a tubular maximum of reabsorption for rat muramidase by the perfused rat kidney observed. It is probable therefore that human and rat muramidase have similar characteristics in the perfused rat kidney, and this adds weight to the observed effects on potassium handling discussed above.

The mechanism of action of muramidase in promoting excretion of potassium is not clear. Although it is concentrated almost exclusively in the cells of the proximal tubule in the present experiments in accordance with earlier suggestions of pinocytosis by renal tubular cells (Hansen *et al*, 1972; Glynn, 1968), this is not the primary site of potassium excretion (Giebisch, 1971). An effect of muramidase on the distal tubule cannot be excluded, since even at low concentrations in the medium, reabsorption was 96% of the filtered load, and some muramidase was detected in the urine, presumably having passed through the distal tubular lumen. The presence of a highly charged molecule in the distal tubular lumen may promote the excretion of potassium in this segment (Giebisch *et al*, 1967). An alternative mechanism for the extra potassium loss by the kidney may be the displacement of potassium from the renal cells by the highly charged muramidase molecule, concentrated within the kidney at a gradient of over 5:1 compared with the perfusion medium. Such a mechanism has been proposed for the potassium excretion induced by some basic amino acids (Dickerman and Walker, 1964) and seen in the isolated perfused rat kidney when 1mM lysine or arginine are added to the medium (unpublished work in this laboratory).

We are grateful to Sarah Bullock, B.Sc. for technical assistance. The work was supported by a research grant from the National Kidney Research Fund.

References

Balazs, T. and Roepke, R. R. (1966). Lysozymuria induced in rats by nephrotoxic agents. *Proc. Soc. exp. Biol. med.* **123,** 380-385.

Dickerman, H. W. and Walker, W. G. (1964). Effect of cationic amino acid infusion on potassium metabolism *in vivo*. *Amer. J. Physiol.*, **206,** 403-408.

Giebisch, G. (1971). Renal potassium excretion. In *The Kidney*, edited by C. Rouiller and A. F. Muller, vol. III, pp. 329-382. Academic Press, New York and London.

Giebisch, G., Klose, R. M., and Malnic, G. (1967). Renal tubular potassium transport. *Bull. schweiz. Akad med. Wiss.*, **23,** 287-312.

Glynn, A. A. (1968). Lysozyme: antigen, enzyme and antibacterial agent. *Sci. Basis Med., Ann. Rev.*, 31-52.

Hansen, N. E., Karle, H., Andersen, V., and Olgaard, K. (1972). Lysozyme turnover in man. *J. clin. Invest.*, **51,** 1146-1155.

Johansson, B. G. and Malmquist, J. (1971). Quantitative immunochemical determination of lysozyme (muramidase) in serum and urine. *Scand. J. clin. Lab. Invest.*, **27,** 255-261.

Maack, T. and Sigulem, D. (1974). Renal handling of lysozyme. In *Lysozyme*, edited by E. F. Osserman, R. E. Canfield, and S. Beychok, pp. 321-333. Academic Press, New York and London.

Mason, D. Y. and Taylor, C. R. (1975). The distribution of muramidase (lysozyme) in human tissues. *J. clin. Path.*, **28,** 124-132.

Muggia, F. M., Heinemann, H. O., Farhangi, M., and Osserman, E. F. (1969). Lysozymuria and renal tubular dysfunction in monocytic and myelomonocytic leukemia. *Amer. J. Med.*, **47,** 351-366.

Osserman, E. F. and Lawlor, D. P. (1966). Serum and urinary lysozyme (muramidase) in monocytic and myelomonocytic leukaemia. *J. exp. Med.*, **124,** 921-952.

Pickering, T. G. and Catovsky, D. (1973). Hypokalaemia and raised lysozyme levels in acute myeloid leukaemia. *Quart. J. Med.*, **42,** 677-682.

Ross, B. D., Epstein, F. H., and Leaf, A. (1973). Sodium reabsorption in the perfused rat kidney. *Amer. J. Physiol.*, **225,** 1165-1171.

Silva, P., Ross, B. D., Charney, A. N., Basarab, H., and Epstein, F. H. (1975). Potassium transport by the isolated perfused kidney. *J. clin. Invest.* (In press.)

Wiernik, P. H. and Serpick, A. A. (1969). Clinical significance of serum and urinary muramidase activity in leukaemia and other haematologic malignancies. *Amer. J. Med.*, **46,** 330-343.

Zucker, S., Hanes, D. J., Vogler, W. R., and Eanes, R. Z. (1970). Plasma muramidase: a study of methods and clinical applications. *J. Lab. clin. Med.*, **75,** 83-92.